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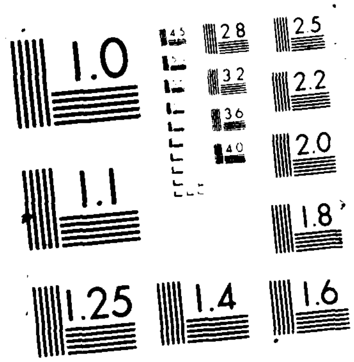
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STUDIES OF ALTERED RESPONSE TO INFECTION INDUCED BY SEVERE INJURY

ANNUAL REPORT

CAROL L. MILLER, PH.D.

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This year has greatly expanded our understanding of the development of immuno-incompetence in trauma patients and suggests the usefulness of clinical trials with PGE_2 inhibitors. Our preliminary data on the relationship between an early (3 days post injury) shift in identifiable MØ subset ratio and the development of immunoincompetence have importance to the rapid detection of patient immuno-incompetence. If we can firmly establish that measurement of the ratio alone is sufficient to predict increased risk of sepsis, then this assessment has potential use in military care of the wounded. A simple assay for determining the ratio of Fc^+ MØ to Fc^- MØ is easily developed. Such an assay would only require drawing a very small blood sample. The peripheral blood could be rapidly segregated on Lymphoprep or other commercial preparations, then MØ isolated by adherence to prepared Akerman Douglas plates for 2 hrs., then labeled with anti Fc floresecent labeled antibody. Counting the number of florescently labeled MØ in a 100 MØ total could then be performed in a very short time resulting in a relevant clinical monitoring tool.

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SUMMARY

This year has greatly expanded our understanding of the development of immuno-incompetence in trauma patients and suggests the usefulness of clinical trials with PGE_2 inhibitors. Our preliminary data on the relationship between an early (3 days post injury) shift in identifiable MØ subset ratio and the development of immuno-incompetence have importance to the rapid detection of patient immuno-incompetence. If we can firmly establish that measurement of the ratio alone is sufficient to predict increased risk of sepsis, then this assessment has potential use in military care of the wounded. A simple assay for determining the ratio of Fc^+ MØ to Fc^- MØ is easily developed. Such an assay would only require drawing a very small blood sample. The peripheral blood could be rapidly segregated on Lymphoprep or other commercial preparations, then MØ isolated by adherence to prepared Akerman Douglas plates for 2 hrs., then labeled with anti Fc florescent labeled antibody. Counting the number of florescently labeled MØ in a 100 MØ total could then be performed in a very short time resulting in a relevant clinical monitoring tool.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).

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For the protection of human subjects, the investigator has adhered to policies of applicable Federal Law 45CFR46.

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Introduction

The goals of this research are (1) to delineate the role of suppressor T cells and inhibitory monocytes (MØ) in mediating the immunosuppression that follows severe injury, (2) to develop and improve assays for assessment of macrophage function, (3) to investigate the relationship between immunosuppression and post-trauma alterations in MØ subsets, and to investigate prophylactic modalities for reversing immunosuppression.

In pursuing goal one of this contract period, we have demonstrated that trauma induced suppressor T cells (T_s) can depress monocyte function and that at least some of the T_s mediate immunosuppression by stimulating monocytes to produce elevated levels of prostaglandin E_2 (PGE_2). In pursuing goal two, we have concentrated on trying to associate a change in MØ function with a change in MØ phenotype. We have shown that elevation of MØ PGE_2 production appears to correlate with the shift toward a MØ subset that can be identified by its expression of a high affinity receptor for the crystaline fragment of Immunoglobulin G (i.e., Fc^+). If it is possible to develop a phenotype profile of a defective MØ, then it would no longer be necessary to do functional assays on patients' blood. Instead, a small quantity of blood could be drawn, the MØ rapidly isolated (2 hours) and stained with fluorescent antibodies which were directed against the surface markers known to identify a particular subset. The increased appearance of this subset could then be enumerated by a simple counting technique allowing quick detection of the onset of immunoincompetence at far forward locations. It is for this reason that we have pursued goal three. We have demonstrated that there is an increase in the numbers of the Fc^+ MØ subset and that this increase correlates both to elevation of PGE_2 production and poor clinical outcome. Finally, in testing of possible new prophylactic

therapies, we have been initially stymied by the failure to have our burned guinea pig model approved by the Animal Research Committee. However, after making several changes in the protocol, we feel that approval should be granted in the next contract year. We have, however, examined the effect of a monocyte stimulator (muramyl dipeptide) on patient MØ function in vitro. Our results suggest that this agent increases MØ PGE_2 production and is contra-indicated in patients who have already undergone a shift in their MØ subset ratios toward a larger proportion of PGE_2 producers. There had been some reports in the literature suggesting the use of MDP as an immunopotentiator.

In summary, this has been a highly productive year in which we have made progress on many of the goals of the five year contract. Our most significant finding is probably the relationship between elevation of immunocompromised patient MØ PGE_2 production and a shift in the patient MØ subset ratios.

Methods

The Plasminogen Activator (PA) assay is performed as previously described (1). The procoagulant assay and the lysozme assay are also performed as previously described (1-2). The assay for suppression of MØ PA activity is performed by isolating the MØ as described (1), then recombining either 2×10^6 normal or 2×10^6 patient MØ with equal numbers (2×10^6) of the patients E-rossetted T cells. The mixed populations are co-cultured for 48 hours in Iscove's supplemented with 8% FCS and media supplements as described (1). In most assays, the normal and patient MØ are collected at day 1-5 post injury and cultured in supplemented Iscove's for 3-4 days. The patient T cells collected at 5-9 days post injury are co-cultured with the normal or patient MØ collected 3-4 days earlier.

MØ PGE_2 is measured with a standard RIA as previously described (3). Leukocyte pyrogen is assessed in a modification of the Bodell assay (4). In this modification, 250 gm Balb/c mice are pre-warmed to 37°, and their baseline temperatures determined over a 20-minute period. The mice then receive a 0.20 ml IV injection of supernates collected from the patients or normal MØ cultures.

The elevation of the mice temperatures is recorded over a 50-minute period with a Yellow Springs Thermoprobe and chart recorder. A media control is also always included.

The sensitivity of MØ to Muramyl Dipeptide is assessed by culturing isolated MØ with 20 μg/ml of MDP for 48 hours in a protocol identical to that we have previously used in experiments with peptidoglycan (5). The PA, PGE_2, Lysozyme, and procoagulant activities of the patient and normal MØ are compared in the presence and absence of MDP. The measurement of the MØ LP levels in the MDP stimulated MØ culture supernates has presented special problems. The correct control is media + MDP. However, both this control and the normal MØ supernates contain residual MDP which in and of itself causes elevation of the mice temperatures. Although the kinetics of this MDP induced pyrogen activity are different than that of LP (LP peak 20 min., MDP peak > 70 min.), the overlap complicated data interpretation.

As previously reported in our quarterly, we now use a protocol whereby the mice are made MDP tolerant by 3 days of injection of increasing MDP doses. When the animals are no longer responsive to MDP, they are used to assess the LP activity of the MDP containing MØ supernates. Using this technique, we have derived significant information on MØ LP stimulation by MDP.

Three methods are used to generate subsets of MØ. All three are rossetting based protocols. In order to isolate MØ positive for the high affinity Fc receptor for IgG, we rossette with anti-Rh antibody coated human erythrocytes using the method of Zembala et al (6). The method for rossetting a population of DQ^+ MØ involves CrCl linking of anti DQ monoclonal antibody to Ox red cells following the method of Mills et al (7). The third method of generating MØ subsets involves treatment of MØ with anti-CR (C3b, C4b receptors) antibody and rossetting the CR negative MØ using anti sheep red blood cell coated erythrocytes with complement compounds 1, 4, 2, 3 on their surface (8).

Results and Discussion

In the period covered by the first year of contract No. DAMD17-86-C-6091, several goals have been attained. Thirty-one patients have been nominated this year. The patients included 19 burn and 11 trauma patients.

Of these patients, twelve were studied the first four months, eight the next three months, seven the third three months, and four the last two months of the year. Of these patients, three burn patients and two trauma patients have succumbed to fatal sepsis. The small number of trauma patients studied is a result of our consistent exclusion of head trauma. Most of the patients are admitted to the trauma center with significant head trauma. Consequently, in the coming year, patients with significant trauma and moderate head trauma will be studied as separate subsets of our trauma patient group.

As we have previously shown, the trauma patients who experienced immunodepression had reduced monocyte (MØ) plasminogen activator (PA) activity with simultaneously elevated MØ PGE_2 and leukocyte pyrogen (LP). As illustrated in Table 1, there is a consistent correlation between (1) MØ PA depression and (2) depression of phytohemagglutinin (PHA) induced T cell melogenics concomitant with (3) PGE_2 elevation of MØ prostaglandin E_2 (PGE_2) levels and (4) poor prognosis. As can be seen, there is little change in MØ PGE_2 in either trauma or burn patients who experience no immunodepression or septic complications. The data showing PGE_2 to be correlated to immunodepression has lead us to re-examine the excessive T lymphocytic suppressor cell activity in immunocompromised trauma patients. We examined the affect of patients' isolated T cells on monocyte responses. Both the patients own and normal monocytes were assessed. The patients' MØ are isolated at day one post injury, then cultured for three days in Iscove's medium. Normal controls are similarly cultured for three days. On day 4-5 post injury, the patients' T cells are isolated and added to both patient and normal cultures. After a further two-day incubation, the response of the normal and patients' MØ without added T cells are compared to the patient and normal cultures which have been incubated two days with

patients' T cells. The patients' T cells collected at 3-7 days post injury consistently suppressed the patients' own MØ plasminogen activator responses, as can be seen in Table 2, Fig. 1. Patients' T cells collected at greater than day 6 post injury consistently suppressed normal MØ PA responses. However, the suppressive activity for normal MØ PA production of patient T cells collected earlier in the post trauma course was variable. These data suggest some genetic restriction on the action of T_s collected at an earlier post injury period. The depression of MØ PA by patient T cells does not appear to result from protein synthesis depression or from cytotoxicity. As also illustrated in Table 2, Fig. 1, and Table 3, the PGE_2 production and leukocyte pyrogen (LP) activity of these immunosuppressed patient MØ are augmented simultaneously to depression of MØ PA activity.

One possible mechanism by which patient T cells could non-specifically suppress normal MØ function is by increasing the MØ PGE_2 levels. In previous years, we have reported an overall increase in PGE_2 production in patients who experienced immunoincompetence. In our recent experiments, we have examined the ability of patients' T_s to increase PGE_2 production. As illustrated in Table 2, Fig. 1, the patient T cells isolated at day 1 post injury are not inhibitory to the PA response nor do they exhibit increased PGE_2 production. However, T cells from the same patient collected at 6-13 days post injury suppressed normal MØ PA, increased MØ PGE_2 levels, and increased normal MØ leukocyte pyrogen (LP) activity. These data imply that increase in MØ PGE_2 production may significantly contribute to immunosuppression of trauma patients. In addition, at least one of the mechanisms for post trauma increases in MØ PGE_2 is the action of T_s on MØ. Increased PGE_2 levels will depress MØ PA production and MØ antigen presenting capacity thereby increasing T_s generation.

As already illustrated, patients' MØ populations exhibit much higher PGE_2 levels than can be induced in normal MØ populations. This apparent increased sensitivity to PGE_2 induction would result if trauma induced (via complement split products, fibrin degradation products or leukocyte mediators) a shift in

the proportion or ratio of the MØ subset that produces PGE_2. Previous investigators have indicated that the MØ population is functionally heterogenous and that this functional heterogeneity is associated with certain MØ subsets. Alternately, trauma could increase the ability of each individual MØ to produce PGE_2.

We have examined the stimulation capacity of Muramyl Dipeptide (MDP) on patient and normal MØ to detect any post-trauma change in MØ responsiveness to bacterial stimuli. MDP is a potent PGE_2 secretagogue and we, and others, have found that it also depresses MØ PA activity. As can be seen in Table 4, 20 μg/ml of MDP stimulates maximal PGE_2 activity in the range of 50,000 pg for both normal and patient day 1 MØ. Increasing the dose of MDP to 30 μg/ml failed to increase the level of PGE_2 above this range. In striking contrast, the patient MØ collected at > Day 6 post were continually producing high levels of PGE_2, and when stimulated with MDP, increased these levels by 2 to 3 fold. The MØ PA responses of these patients already appeared to be maximally suppressed and did not additionally decrease their PA and responses upon MDP stimulation. These data indicate either a post-trauma increase in a PGE_2 producing subset or an increased capacity for PGE_2 production in all MØ.

We examined the subset ratios of patients MØ populations using the MØ subset with a high affinity receptor for IgG2 (Fc^+). This Fc^+ subset has been previously suggested to contain the inhibitory MØ population (6). We, therefore, examined the PGE_2 production and Fc^+ subset proportions in immunosuppressed trauma patients. As can be seen in Figures 2 and 3, immunodepressed patients had a dramatic increase in the proportion of the Fc^+ MØ which coincided with an increase in their PGE_2 activity. The Fc^+ MØ were the primary, if not the sole, MØ producers of PGE_2. The normal MØ and immunocompetent patient MØ populations contained approximately 40% high affinity Fc^+ MØ and 60% Fc^- MØ after isolation on Ackerman Douglas Flasks. We have now examined a number of patients over time post injury. Those patients who survived multiple septic episodes and regained immunocompetence showed a return

to normal Fc^+/Fc^- MØ ratio levels (Table 5). Trauma patients with normal PA responses had normal Fc^+/Fc^- MØ ratios. Interestingly, patients with depressed MØ PA had normal PA responses when only their isolated Fc^- monocytes were assessed in the absence of the Fc^+ subset. Similarly, the patients late T_s were unable to suppress either the patients Fc^- or normal Fc^- MØ PA responses. Only Fc^+ responses were suppressed (Table 6). These data support the idea that some of the T_s suppression mechanism may be partially a result of increasing MØ PGE_2 activity.

We have also examined the leukocyte pyrogen (LP) responses of the Fc^+ and Fc^- subsets and found that LP activity is confined primarily to the Fc^+ MØ subset (Table 7). Addition of immunosuppressed patient T_s to normal or day 1 post injury patient MØ was found to increase the MØ LP activity as well as depress the MØ PA activity.

In summary, these data further support our hypothesis that a shift in the trauma patient Fc^+ to Fc^- MØ ratio increases overall patient PGE_2 production. Furthermore, excessive T_s generation may also contribute to elevating MØ PGE_2 levels and development of immunoincompetence. Several questions on the mechanism by which trauma could mediate immunosuppression are currently being explored. It is possible that immunosuppressive patient Fc^- MØ are altered so that they can respond to MDP with PGE_2 production. We have already shown that normal Fc^- monocytes can not be stimulated by MDP to produce PGE_2 or LP. However, we do not as yet have data indicating that patient Fc^- MØ do not respond to MDP stimulation by increasing their PGE_2 or LP output. It also must be noted that the MØ subset we are calling Fc^- is not, in fact, devoid of Fc receptors. The anti RH antibody consists primarily of IgG2. Human monocytes have two receptors for IgG, a "high affinity" receptor with a preference for IgG2, and a "low affinity" receptor with a preference for IgG_1. When we utilized a specific anti low affinity Fc receptor antibody, we found that most (if not all) MØ have the low affinity Fc receptor, but only a subset express the high affinity. Consequently, the Fc^+ population we are describing in actuality

is the high affinity positive population. Nevertheless, our data show that this high affinity positive MØ subset is increased at the expense of the low affinity (Fc^-) subset in immunocompromised trauma patients. This high affinity Fc^+ MØ subset is enriched for both LP and PGE_2 producers but not for PA producers. The result of this shift in MØ subset Fc^+/Fc^- ratio is that patients who develop immunoincompetence show increased PGE_2 production and respond to bacterial stimuli (MDP) with a greater production of PGE_2 than normal individuals' MØ. Furthermore, T cells from immunoincompetent patients can depress facilitory MØ functions (i.e. PA) and increase MØ PGE_2 levels thereby further perpetuating the immunosuppressive cycle.

This year has greatly expanded our understanding of the development of immuno-incompetence in trauma patients and suggests the usefulness of clinical trials with PGE_2 inhibitors. In addition, these preliminary data are on the relationship between an early (3 days post injury) shift in identifiable MØ subset ratio and the development of immunoincompetence have importance to the rapid detection of patient immunoincompetence. If we can firmly establish that measurement of the ratio alone is sufficient to predict increased risk of sepsis, then this assessment has potential for army use. A simple assay for determining the ratio of Fc^+ MØ to Fc^- MØ is easily developed. Such an assay would only require drawing a very small blood sample. The peripheral blood could be rapidly segregated on Lymphoprep or other commercial preparations, then MØ isolated by adherence to prepared Akerman Douglas plates for 2 hrs., then labeled with anti Fc florescent labeled antibody. Counting the number of florescently labeled MØ in a 100 MØ total could then be performed in a very short time resulting in a relevant clinical monitoring tool.

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TABLE 1

Correlation MØ and T Cell Depression and Clinical Outcome

Pt	Injury	ΔPHA%[a]	PA(01-13)[b]	$\Delta PGE_2 \times 10^{-3}$ pg[c]	Outcome
Ja	Trauma	55	41.8 → 14.1	1.9 → 15.4	Multiple septic episodes
Sw	Trauma	- 90	26.8 → 8.6	1.5 → 12.8	Fatal sepsis
Ch	Trauma	- 20	34.1 → 22.0	8.4 → 6.8	No complications
Br	Trauma	- 15	18.0 → 22.2	4.3 → 1.6	No complications
Pr	Trauma	- 20	32.9 → 24.4	12.3 → 12.3	No complications
Mu	Burn	- 87	48.8 → 7.2	1.6 → 23.6	Fatal sepsis
Ke	Burn	- 59	32.1 → 12.9	2.7 → 11.3	Multiple septic episodes
Ar	Burn	- 82	45.4 → 19.1	5.9 → 14.2	Multiple septic episodes
Be	Burn	- 79	41.6 → 15.0	5.3 → 12.5	Fatal sepsis
Ho	Burn	- 80	39.2 → 8.2	3.3 → 13.9	Multiple septic episodes
Cr	Burn	- 78	31.8 → 14.7	2.6 → 11.1	Fatal sepsis
Gh	Burn	+ 30	44.2 → 39.9	5.8 → 5.0	No complications
Jo	Burn	+100	26.1 → 21.5	7.0 → 8.8	One infectious episode
Ro	Burn	+ 12	32.4 → 27.6	2.8 → 2.6	No complications
Nu	Burn	- 20	25.0 → 18.9	6.7 → 9.9	No complications

a. Maximum change at 4-13 days post injury in mitogen induced proliferation of 2×10^5 peripheral blood mononuclear cells to 0.2 mg PHA

b. Maximum change in MØ plasminogen activator (PA) activity from initial response to most depressed in the first 13 days post injury. PA activity measured in % plasmin specific fibrinolysis

c. Maximum prostaglandin E_2 production in first 13 post injury days measured as picograms per 10^6 recovered MØ ($\times 10^{-3}$)

TABLE 2

EFFECT OF PT T_s-CELLS ON MØ FUNCTION [a]

PGE_2	$pg/10^6$MØ [b]	PA
Norm MØ + D1 PT T-Cells[c]	9,025	30.4
Norm MØ	13,544	27.1
Norm MØ + D>6 T-Cell	45,451	16.5
Norm MØ	22,610	34.5

a. T lymphocytes were isolated from patient mononuclear preparations by E rossetting of MØ depleted populations.

b. PGE_2 in picograms per 10^6 recovered MØ.

c. 2 x 10^6 MØ were cocultured 2 days with 2 x 10^6 patient (pt) T lymphocytes collected at day 1 post-injury (D1).

Table 3

EFFECT OF PATIENT T CELLS ON MØ FUNCTION

CELLS	LP Δtemp[a] Exp 112	LP Δtemp[a] Exp 130	PA%[b] Exp 112	PA%[b] Exp 130
Norm MØ	.3	.15	59.5	52.6
Norm MØ + Pt T Cell[c]	.35	.20	37.7	22.3
Pt MØ	.35	.65	21.5	49.9
Pt MØ + Pt T Cell[d]	.75	.85	10.5	13.9

a. Leukocyte pyrogen (LP) was assessed as change in temperature (Δtemp) of mice injected with 0.3 ml MØ supernate

b. MØ production of plasminogen activator (PA) is measured in percent specific fibrinolysis

c. $2x10^6$ normal individual's MØ were cocultured for 2 days with $2x10^6$ patient (Pt) T lymphocytes

d. $2x10^6$ patient MØ isolated at day 1-3 post-injury and maintained in culture were then cocultured for 2 days with the Pt T cells collected 4-6 days post-injury

Table 4

EFFECT OF MDP[A] ON NORMAL OR PATIENT MØ FUNCTION

Mo	PGE_2[b]	PA[c]
Norm	16 944 ± 6 717	31.4 ± 7.4
Norm + MDP	54 387 ± 23 030	15.2 ± 3.8
D1 Pt	19 759 ± 7 295	33.5 ± 12.1
D1 Pt + MDP	49 049 ± 22 529	13.8 ± 4.2
D>6 Pt	37 940 ± 11 606	14.4 ± 4.1
D>6 Pt + MDP	91 851 ± 20 951	14.0 ± 1.6

A. 2×10^6 MØ CULTURED FOR 2 DAYS WITH 20 µG/ML OF MURAMYL DIPEPTIDE (MDP)

B. PROSTAGLANDIN E_2 PRODUCTION IN PICOGRAMS PER 10^6 RECOVERED MØ

C. PLASMINOGEN ACTIVATOR ACTIVITY IN % SPECIFIC FIBRINOLYSIS.

TABLE 5

Change on MØ Fc^+/Fc^- subset ratios over time post injury

	% Fc^+/Fc^-		Fc^+	Fc^-
PtT day 15	75/25		38±6	62±6
day 21	66/34	X Normal = 14		
day 29	80/20			
day 37	58/42			
day 47	48/52			
day 52	47/53			
PtR day 9	62/38			
day 16	32/68			

Table 6

PA Activity of MØ subsets after interaction with patient T cells

	Unsep[a]	Fc+[b]	Fc^-[c]	Unsep + T[d]	Fc^-+T	Fc^++T
Pt	43.8	32.9	59.3	-	-	-
Nor	49.6	29.4	41.6	-	-	-
Pt	34.1	38.3	41.9	-	-	-
Nor	26.1	27.0	41.8	-	-	-
Pt	10.9	8.2	19.7	-	-	-
Nor	19.3	11.5	29.5	-	-	-
Pt	19.4	22.6	31.5	-	-	25.2
Nor	21.7	22.4	33.5	-	29	30.1
Pt	31.7	20.6	46.4	18.6	29.3	6.9
Nor	26.5	21.4	35.4	-	-	19.2

a. Plasmin specific fibrinolysis of 5×10^5 unseparated (Unsep) heterogenous MØ from either patients (Pt) or Normals (Nor)

b. 5×10^5 $MØ^-$ with high affinity receptors for IgG rossetted with anti-RH antibody coated human erythrocytes

c. 5×10^5 MØ depletion of Fc^+ rossetting cells

d. 5×10^5 MØ derived from cultures incubated with Pt T lymphocytes collected at 3-4 days after the MØ

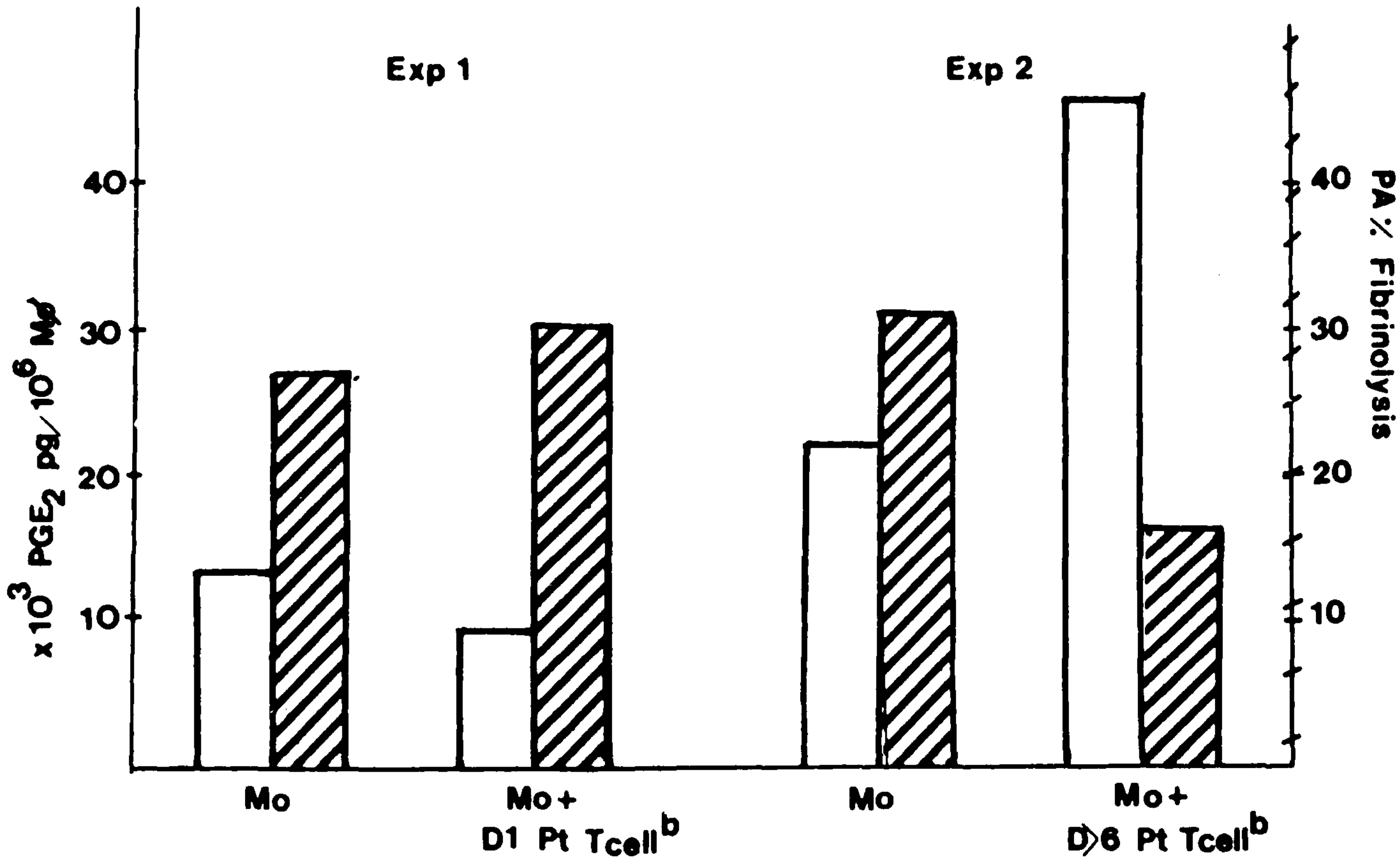
Table 7

Fc^{+} MØ Subset Contains the MØ which are Producing Leukocyte Pyrogen (LP)

MØ Pop[A]	Patient		Normal	
	PGE_2	LP[B]	PGE_2	LP
Fc^{+}	12,665	.55	4,058	.25
Fc^{-}	1,484	0	847	0

A. MØ populations (Pop) were selected for Fc receptor by rossetting with anti Rh coated red cells.

B. Leukocyte pyrogen was assayed as change in temperature (Δ Temp) of mice injected with 0.3 ml of MØ supernate.

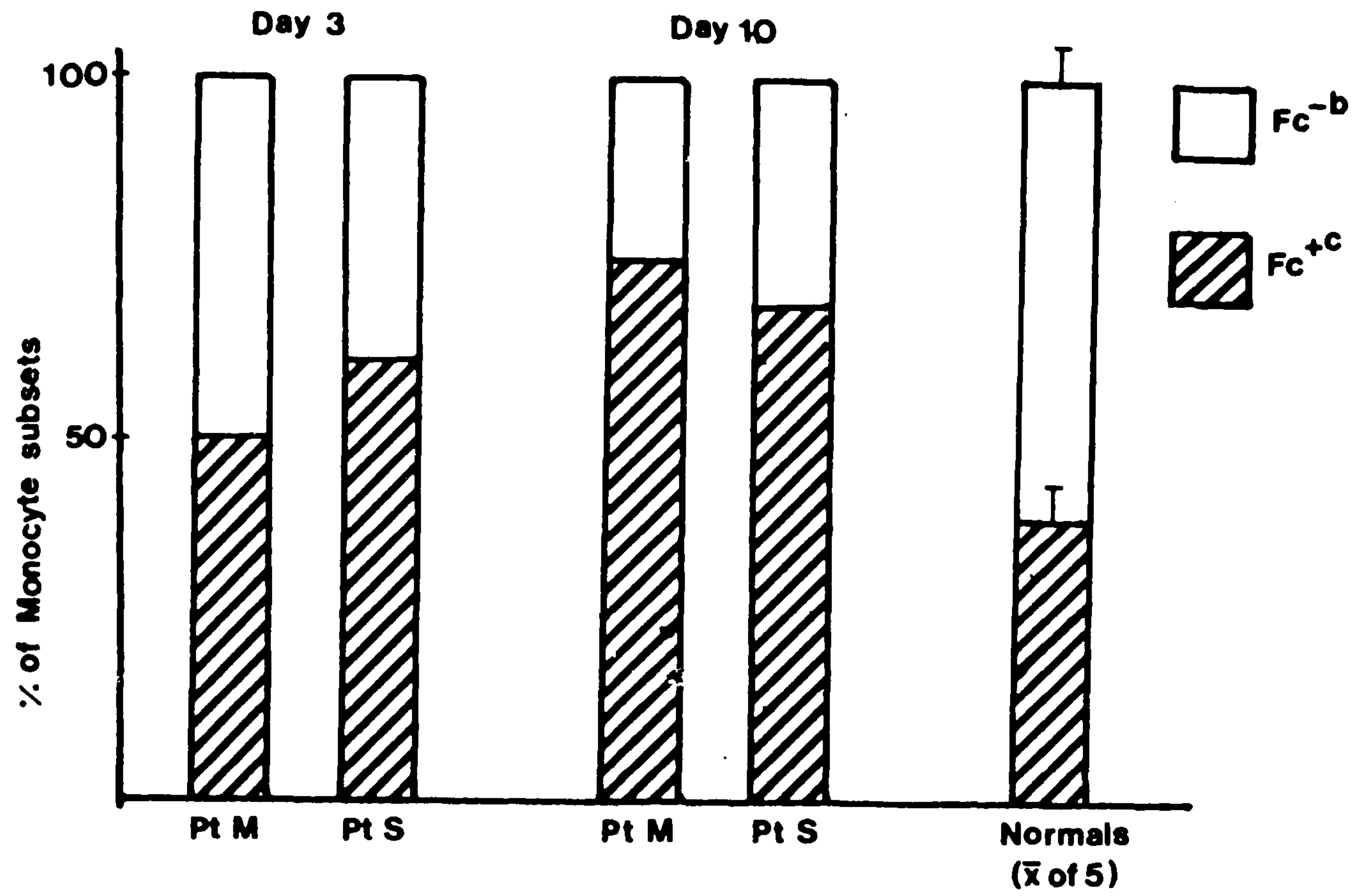


A. T LYMPHOCYTES WERE ISOLATED FROM PATIENT MONONUCLEAR PREPARATIONS BY E ROSETTING OF Mø DEPLETED POPULATIONS

B. $2x10^6$ NORMAL Mø WERE COCULTURED FOR 2 DAYS WITH $2x10^6$ PATIENT T LYMPHOCYTES

FIG. 1

EFFECT OF PATIENTS' T CELLS[A] ON NORMAL Mø FUNCTIONS



A. Isolation of patient Fc^{+} and Fc^{-} Mo populations performed by rosetting with anti-Rh antibody coated human erythrocytes

B. Non rosetting population

C. Percent of the total population rosetting with anti-Rh coated erythrocytes

FIG. 2

INCREASE IN Fc^{+} MØ POPULATION IN IMMUNOINCOMPETENT PATIENTS[A]

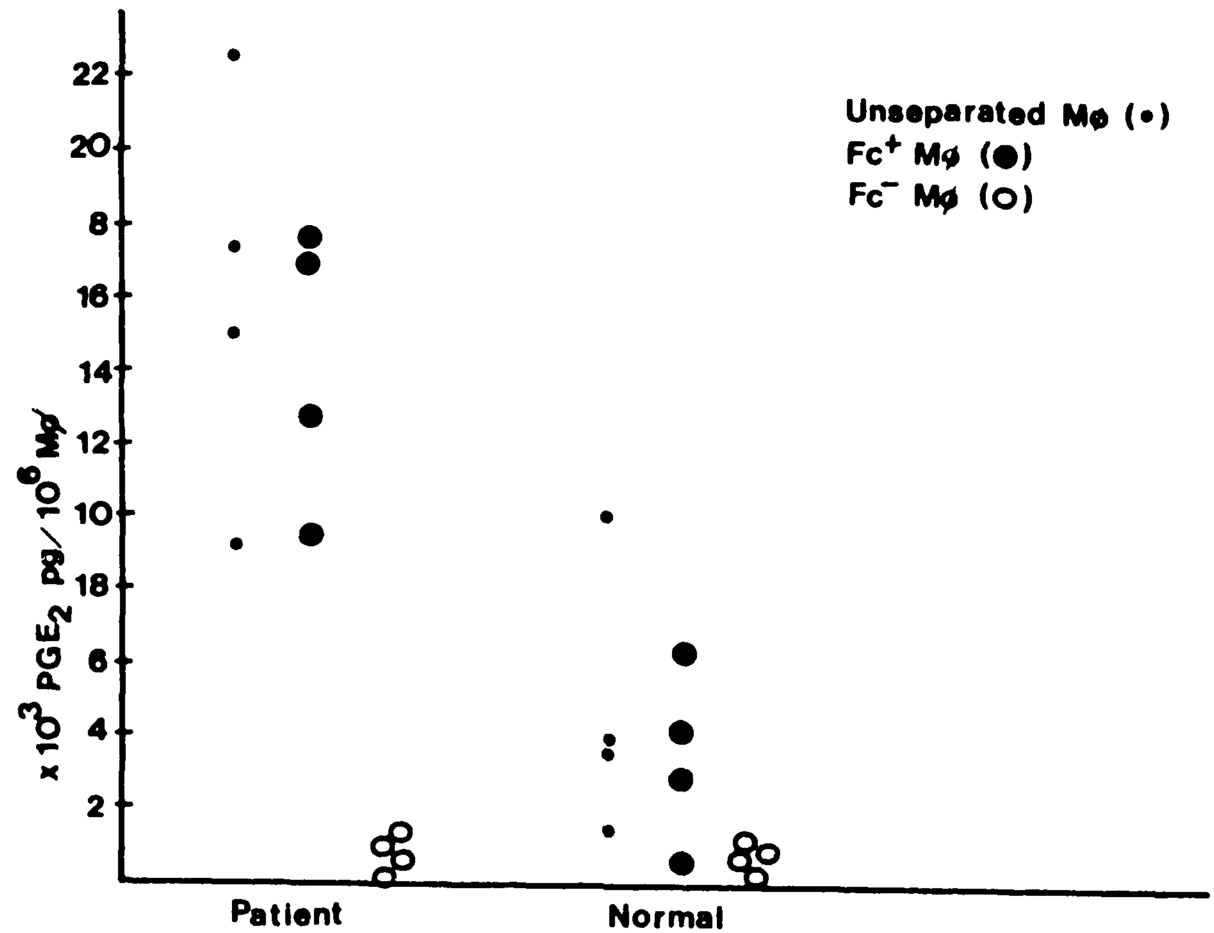


FIG. 3

Fc$^+$ MØ SUBSET[A] CONTAINS THE PGE_2 PRODUCING CELLS

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